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Mechanistic Insights into the Modulation of Lipopolysaccharide-Stimulated Inflammatory and Oxidative Stress Responses by *Myrmecodia platytyrea* Tuber Ethyl Acetate Extract in RAW 264.7 Macrophages

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ABSTRACT

Southeast Asians frequently utilise *Myrmecodia platytyrea* (Rubiaceae) to treat inflammatory diseases, but little is known about its molecular mechanisms. We evaluated the effect of *M. platytyrea* tuber ethyl acetate extract (EAMPT) on lipopolysaccharide (LPS)-induced inflammation and oxidative stress in RAW 264.7 cells. The total phenolic content of EAMPT was determined using high-performance thin-layer chromatography (HPTLC). *In vitro*, the RAW 264.7 cells were divided into four groups: control (untreated), LPS (1 µg/mL LPS), treatment (3.75 to 60 µg/mL EAMPT plus LPS), and reference (200 µM ibuprofen plus LPS). Subsequently, cell culture samples were tested for cell viability, oxidative stress, and inflammatory markers using the MTT assay, biochemical assays, ELISA, real-time qPCR, and Western blot. EAMPT, with a total phenolic content of 4.15%, suppressed LPS-induced inflammation by lowering gene and protein expression of the iNOS/NO pathway and TNF-α. Furthermore, ROS levels decreased, while HO-1 gene and protein expression increased, suggesting the antioxidant effect of EAMPT. The ROS-inhibitory effect was strongly correlated with the NO-inhibitory effect. EAMPT increased IL-6 gene and protein expression, while no effect on the COX-2/PGE2 pathway was observed. In summary, EAMPT offers synergistic effects through dual mechanisms in an *in vitro* inflammation model, highlighting iNOS and HO-1 as therapeutic targets. Further research on *M. platytyrea* tuber's pharmacological and phytochemical characteristics may reveal its novel therapeutic potential.

Keywords: Ant nest tuber, sarang semut, immunomodulation; phenolics; macrophages, *Myrmecodia*

INTRODUCTION

Chronic inflammation is characterised by an imbalance of cellular redox state and continuous generation of inflammatory mediators, resulting in tissue damage and failed immune response. Some inflammatory-related diseases include coronary artery diseases (Sardu *et al.,* 2020), cancers (Piotrowski *et al.,* 2020), rheumatoid arthritis (Maeda *et al.,* 2022), inflammatory bowel disease (Massironi *et al.,* 2023), neurodegeneration such as Alzheimer's (Simpson & Oliver, 2020), and metabolic disorders (Lee & Olefsky, 2021). According to a recent Global Burden of diseases study, the age-standardised incidence

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rate of six immune-mediated inflammatory diseases has increased globally in the low-middle sociodemographic index countries in the past 30 years (Wu *et al.,* 2023). Despite advances in pharmacotherapy, current treatment options often have adverse effects and may not fully address the complex, multifaceted pathophysiology of inflammation. Thus, continuous research is urgently needed to develop a safe and effective pharmacotherapy targeting the molecular mechanisms of inflammation.

One of the underlying triggers of chronic inflammation is oxidative stress. It is an imbalance of the cellular redox system caused by the overproduction of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide anion, as well as reactive nitrogen species (RNS), such as nitric oxide (NO). Invasion by pathogens (viruses, microbes, and parasites), allergens, or other injurious agents causes an increase in ROS and RNS production, which activates inflammatory signalling pathways and the subsequent release of various inflammatory mediators and cytokines (Hussain *et al.,* 2016). Notably, in an acute inflammatory response, lipopolysaccharide (LPS) classically activates the macrophages, which polarise to the M1-type and generate excessive amounts of ROS, RNS and cytokines to aid in the recruitment of T-cells. M1 macrophages are antigen-presenting cells that play a vital role in the innate immune system, with the mission to kill pathogens and repair inflammation. However, regulating M1 macrophages is critical in preventing excessive tissue damage in the context of persistent inflammatory stimuli or inadequate resolution of acute inflammation, which could progress to a chronic inflammatory state (Atri *et al.,* 2018).

Plants have been traditionally used as natural remedies against inflammation and oxidative stress for centuries. The presence of bioactive secondary metabolites, including polyphenols, flavonoids, alkaloids, and terpenoids, may exhibit various therapeutic activities with diverse modes of action. Moreover, these natural antioxidant compounds exert their immunomodulatory potential by scavenging ROS and RNS to prevent oxidative stress, thereby suppressing inflammation. Hence, herbal plants and fruits, such as those containing polyphenols with known significant antioxidant activities, are commonly used as traditional medicine for treating inflammation-related diseases (Rudrapal *et al.,* 2022). *Myrmecodia platytyrea* is the scientific name of an ant-nest plant (also locally known as sarang semut) from the Rubiaceae family, commonly found in the Pacific Islands, Papua, and Southeast Asia regions, including Malaysia, Indonesia, and the Philippines. Unlike other *Myrmecodia* species characterised by pale or white tuber flesh, *M. platytyrea* exhibits a distinctive red pigmentation within its tuber parenchyma, suggesting a unique phytochemical profile with potential implications for species-specific bioactivity and ecological adaptation. Phytochemical studies identified various groups of natural compounds from the *Myrmecodia* genus – namely, flavonoids, tannins, alkaloids, saponins, iridoids and steroids (Dirgantara *et al.,* 2022). *M. platytyrea* extracts showed excellent antioxidant potential, with the ethyl acetate fraction being the most effective in 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (half maximal effective concentration [EC_{50}] = 32.91 ± 2.23 μg/mL) (Mohamad Haris *et al.,* 2013). Morindolide, a rare iridoid lactone, was isolated from the ethyl acetate extract of *M. platytyrea*, which might also contribute to its antioxidant potential (Mohamad Haris *et al.,* 2016). Several preclinical studies have demonstrated Rubiaceae plants' anti-inflammatory properties, such as *Gardenia jasminoides* Ellis, *Morinda officinalis* How, and *Paederia scandens*. The pharmacological effect may be attributed to the mixtures of iridoid compounds in the plant extracts, including genipin, geniposide, and monotropein (Jaafar *et al.,* 2023). On top of that, the aqueous tuber extract of *M. platytyrea* was nontoxic to mice based on an acute and subacute toxicity study (Mohd Zin *et al.,* 2024). Given its phytochemistry, biological activities, and toxicity profile, *M. platytyrea* extracts could be a promising source of natural compounds with important medicinal values.

While modern pharmacotherapy remains a cornerstone in managing inflammation, there is a growing interest in exploring alternative medicines derived from natural sources. Plant-derived therapeutic agents have demonstrated a reasonably safe profile and greater accessibility, with the potential for synergistic effects that target numerous signalling pathways implicated in the interplay between inflammation and oxidative stress. To the best of our knowledge, this will be the first study to investigate the effects of ethyl acetate extract of *M. platytyrea* tubers (EAMPT) on LPS-induced inflammation and oxidative stress in mouse Raschke, Watson, and Ralph (RAW) 264.7 cells, and whether these effects are correlated to its

antioxidant effect and phenolic content is unknown.

MATERIALS AND METHODS

Plant material and extraction

The *M. platytyrea* tubers were collected from Sulawesi, Indonesia and authenticated by Prof. Dr. Eko Baroto (Herbarium Bogoriense Research Centre for Biology, Indonesia). The voucher specimens (BO1647929 and BO 0009642) were deposited at the herbarium. The dried tubers were macerated in an analytical-grade ethyl acetate (EMSURE®, Sigma-Aldrich, USA) for 24 h at 1:9 (w/v) to prepare the extract. Then, the crude extract was filtered, and the solid residue was re-soaked for a second time in fresh ethyl acetate for another 24 h before filtering again. The filtrates were pooled, and the solvent was evaporated at 40°C and 150 mbar using a rotary evaporator (Heidolph, Germany). The dried crude extract was then collected and stored in an airtight container at -80°C for long-term use. The average extraction yield calculated from three independent macerations of 250 g raw *M. platytyrea* tubers was 3.09 ± 0.32 g, corresponding to 1.23 ± 0.11% of the starting raw material as previously described in an earlier preliminary study (Jaafar *et al.,* 2024).

Determination of total phenolic content

The total phenolic content of EAMPT was determined using a CAMAG® high-performance thin-layer chromatography (HPTLC) system (CAMAG, Muttenz, Switzerland) based on a validated method (Ghafari *et al.,* 2021). An HPTLC glass plate with silica gel 60 F254 (Merck, Germany) was pre-washed with 100% high performance liquid chromatography (HPLC)-grade methanol (Merck, Germany) and left to dry at 100°C for 30 min. Sample and standard solutions were prepared using HPLC-grade methanol at 10 mg/mL for EAMPT and 1 mg/mL for gallic acid, respectively. Standard solutions were applied at five different volumes to construct a calibration curve. A two-step gradient elution mobile phase development was performed: 100% methanol over 40 mm of developing distance, followed by n-hexane, ethyl acetate, and acetic acid (6.7:3:0.3, *v/v*) over 80 mm of developing distance. The developed plate was photo-documented under white light, ultraviolet (UV) light, and 254 nm wavelength and derivatised with 2% ferric chloride solution for 1 s. The plate was left to dry at 100°C for 10 min and photo-documented under daylight. For quantitative determination of phenols, the plate was scanned with the thin layer chromatography (TLC) Scanner 3 (CAMAG, Muttenz, Switzerland) at 20 mm/s scanning speed, and WinCATS version 1.4.9.2001 (CAMAG, Muttenz, Switzerland) was used to perform the densitogram analysis.

RAW 264.7 cell culture and study design

A murine macrophage RAW 264.7 cell line was obtained from the American Type Culture Collection (ATCC) (Virginia, United States) and cultured in a High Glucose-Dulbecco's Modified Eagle's Medium (HG-DMEM) (Nacalai Tesque, Japan) supplemented with 10% foetal bovine serum (Tico Europe, Netherlands) and 1% penicillin-streptomycin (Nacalai Tesque, Japan). The cell culture was maintained in a humidified atmosphere with 5% carbon dioxide (CO_2) at 37°C and passaged every two days using the trypsinisation method. For experimental purposes, RAW 264.7 cells were grown in an appropriate culture flask and left overnight to achieve 70% confluence the next day. Subsequently, the cells were pre-treated either with or without EAMPT at various concentrations, or ibuprofen (IBP) (Sigma-Aldrich, USA) at 200 µM as the reference drug for 1 h, followed by induction with 1 µg/mL LPS (from *Escherichia coli* serotype O55:B5) (Sigma-Aldrich, USA) for an additional 24 h at 37°C with 5% CO^2. For cell-based assays and enzyme-linked immunosorbent assay (ELISA), the concentrations of EAMPT used for treatment were 3.75, 7.5, 15, 30, and 60 µg/mL, as the extract's half-maximal inhibitory concentration (IC_{50}) on the non-activated RAW 264.7 cell line was found to be 60 µg/mL (Jaafar *et al.,* 2024). Meanwhile, only two concentrations of EAMPT (30 and 60 µg/mL) were utilised for gene and protein expression analyses, as these concentrations showed notable influence on the parameters investigated. In addition, LPS incubation was reduced to 4 h for quantitative polymerase chain reaction (qPCR) experiments to evaluate gene expression earlier compared to protein expression at 24 h.

Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was employed to determine the effect of EAMPT on the viability of LPS-stimulated RAW 264.7 cells. The assay was performed as previously described (Riss et al., 2013) by incubating the cells with MTT reagent (Sigma-Aldrich, USA) for 2 h. After incubation, the supernatant in each well was

discarded and replaced with 100 µL of analytical-grade dimethyl sulfoxide (DMSO) (Fisher Scientific, UK) to dissolve the insoluble formazan crystals. The Infinite M200 Pro microplate reader (Tecan Life Sciences, Switzerland) was used to measure the absorbance of each well at 570 nm. The viability of the control cells was standardised to 100%, and the relative viability for cells treated with EAMPT or IBP and LPS was calculated using the following equation:

$$\text{Cell viability (\%)} = \frac{(AS\text{-}AB)}{(AC\text{-}AB)} \times 100$$

Where, AS = absorbance of extract- or drug-treated cells, AC = absorbance of DMSO-treated cells, AB = absorbance of blank (media only).

Measurement of intracellular ROS

The generation of intracellular ROS was assessed using the 2,7-dichlorofluorescein-diacetate (DCFH-DA) assay, a fluorescent indicator sensitive to ROS. DCFH-DA diffuses into live cells and is broken down by cellular esterase to produce DCFH. ROS within the cells interacts with DCFH to produce a highly fluorescent DCF molecule. LPS-stimulated RAW 264.7 cells pre-treated with or without EAMPT or IBP were washed twice with 1× phosphate-buffered saline (PBS), including the control cells. Then, the cells were incubated with 100 µL of 10 µM Highly Sensitive DCFH-DA dye (Dojindo, Japan) at 37°C with 5% CO^2 for 30 min. Fluorescent images were viewed and captured at 100X magnification with a green fluorescent protein (GFP) filter using the inverted fluorescence microscope (Nikon, Japan). The mean fluorescence intensity of each image was analysed using ImageJ (National Institutes of Health, USA).

Griess assay

The NO level was measured based on the Griess reaction, which quantifies the nitrite level in cell culture supernatant. Culture supernatants of LPS-stimulated RAW 264.7 cells pre-treated with or without EAMPT or IBP, including those of control cells, were collected into a new 96-well plate. Fresh culture medium was included as the blank control. Based on the instruction manual of Griess Reagent System (Promega Corporation, USA), 50 µL of standard solution and samples were mixed with 50 µL of Sulphanilamide Solution and 50 µL of *N*-(1-naphthyl)ethylenediamine dihydrochloride (NED) Solution. The absorbance was measured using the Infinite M200 Pro microplate reader (Tecan Life Sciences, Switzerland) at 540 nm. The NO level in each sample was extrapolated from the standard curve constructed.

Enzyme-linked immunosorbent assay

The concentrations of inflammatory markers, namely, tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), and prostaglandin E2 (PGE2) in LPS-induced RAW 264.7 cells pre-treated with EAMPT or IBP, were determined using ELISA method. Commercial ELISA kits for the detection of mouse TNF-α, IL-6, and PGE2 (Elabscience, USA) were used according to the manufacturer's protocol. The absorbance readings were measured at 450 nm using the Infinite M200 200 Pro microplate reader (Tecan Life Sciences, Switzerland).

Gene expression analysis

The total RNA from cell lysates was isolated using the RNEasy Plus Kit (Qiagen, Germany) based on the spin column method. The concentration and purity of the extracted RNA were determined using a QIAxpert spectrophotometer (Qiagen, Germany). Then, 1 µg of total RNA was reverse transcribed to complementary deoxyribonucleic acid (cDNA) using the QuantiTect Reverse Transcription Kit (Qiagen, Germany) according to the manufacturer's instructions. For analysing the expression of the target genes, equal amounts (50 ng) of cDNA were subjected to real-time qPCR with QuantiNova SYBR Green PCR Kit (Qiagen, Germany) and primers using a Rotor-Gene Q real-time PCR cycler (Qiagen, Germany) (Table I).

Table I. Primer sequences used in this study

Gene	Sequence ('5 → ' 3)
TNF-α	F- GCGACGTGGAACTGGCAGAA
	R- CAGTAGACAGAAGAGCGTGGTG
IL-6	F-CGCACTAGGTTTGCCGAGTA
	R- CCTTTCTACCCCAATTTCCA
iNOS	F- CAGCGGAGTGACGGCAAACAT
	R- GCAAGACCAGAGGCAGCACATC
COX-2	F- TGGGTGTGAAGGGAAATAAGG
	R- CATCATATTTGAGCCTTGGGG
HO-1	F- CACGCATATACCCGCTACCT
	R- CCAGAGTGTTCATTCGAGCA
GAPDH	F- CAAAATGGTGAAGGTCGGTG
	R- CGTTGATGGCAACAATCTCC

Abbreviations: TNF-α (tumour necrosis factor-α); IL-6 (interleukin-6); iNOS (inducible nitric oxide synthase); COX-2 (cyclooxygenase-2); HO-1 (heme oxygenase-1); GAPDH (glyceraldehyde 3-phosphate dehydrogenase).

The thermocycling condition was programmed as follows: PCR initial heat activation at 95°C for 2 min, two-step cycling consisting of a denaturation step at 95°C for 5 s and a combined annealing/extension step at 60°C for 10 s for 40 cycles, and melting curve analysis. The relative gene expression of the target genes was determined by normalisation to GAPDH as the internal control using Livak's method ($2^{-\Delta\Delta Ct}$) (Livak & Schmittgen, 2001).

Western blot analysis

Cell samples were washed with ice-cold 1× phosphate-buffered saline, lysed in radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, USA) supplemented with 1% phenylmethylsulfonyl fluoride (Santa Cruz Biotechnology, USA) and 1% phosphatase inhibitor cocktail (Santa Cruz Biotechnology, USA), and sonicated on an ice water bath for 15 min. Total protein content was determined using Pierce™ Bradford Protein Assay Kit (Thermo Fisher Scientific, USA). Equal amounts of protein samples (30 µg of protein) were boiled in 5× sodium dodecyl sulfate (SDS) Loading Buffer (Elabscience, USA), separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 h and incubated overnight with primary antibodies against inducible nitric oxide synthase (iNOS) (GTX130246, GeneTex, USA), cyclooxygenase-2 (COX-2) (GTX100656, GeneTex, USA), heme oxygenase-1 (HO-1) (E-AB-81442, Elabscience, USA), and β-actin (GTX629630, GeneTex, USA). The membranes were washed with TBS-T thrice and incubated with horseradish peroxidase-conjugated secondary antibodies (Elabscience, USA) for 1 h. After three washes in TBS-T, the protein bands were incubated with Radiance Q chemiluminescent reagent (Azure Biosystems, USA) for 5 min and visualised with a C-Digit Blot Scanner (LI-COR Biosciences, USA).

Statistical analysis

Data are reported as the mean ± standard error of the mean (SEM) from at least three independent experiments performed in triplicate. GraphPad Prism version 9 (GraphPad Inc., USA) was used to evaluate statistical significance by one-way analysis of variance (ANOVA), with a p-value of less than 0.05 deemed statistically significant. Dunnett's post-hoc test was then used to determine the statistical significance between the experimental and control groups in a specific experiment. A correlational analysis was applied to investigate the relationship between the two main variables in this study based on Pearson's method. A significant positive relationship is suggested when the correlation coefficient value between the two variables is close to +1, while a strong negative association is indicated when the value is close to -1. If the r value is zero, there could not be a linear relationship between the two variables.

RESULTS AND DISCUSSION

Total phenolic content in EAMPT

HPTLC analysis indicated an RF (retention factor) value of 0.47 ±0.01 for gallic acid. The presence of phenols in EAMPT was confirmed when the sample track showed a band with an RF value of 0.474, corresponding to the standard gallic acid value shown in the chromatograms (Figure 1). Furthermore, the sample bands at RF = 0.474 were stained dark green upon derivatisation with 2% ferric chloride, confirming the presence of phenols. A five-point calibration curve (Supplementary 1) constructed for the analysis using gallic acid (concentrations from 2 to 6 µg/band) follows a linear equation where $y = 0.0018x + 0.0012$. The R2 value of the linear curve was 0.9774, demonstrating high reliability of the calibration curve for predicting phenolic concentration. Hence, based on this method, the total phenolic content of EAMPT was 41.53 ± 2.87 mg gallic acid equivalent per g of extract's dry weight (mg GAE/g dw), equivalent to 4.15% (w/w).

The *Myrmecodia* genus has been traditionally used in Southeast Asian communities for its anti-inflammatory and anticancer properties, which may be due to its antioxidant potential. Our findings revealed that EAMPT contains 4.15% phenolic content, which is considered relatively significant compared to published findings (Kähkönen *et al.,* 1999). The range of phenolic content is classified into three categories: low (≤ 10 mg GAE/g dw), moderate (11 – 20 mg GAE/g dw) and high (≥ 40 mg GAE/g dw) (Stanojević *et al.,* 2009). This could reflect the potential therapeutic activities of EAMPT against LPS-induced inflammation and oxidative stress in the present study. Some major phenolic compounds previously identified from the *Myrmecodia* genus include luteolin, rutin, kaempferol, apigenin, and gallic acid (Dirgantara *et al.,* 2022).

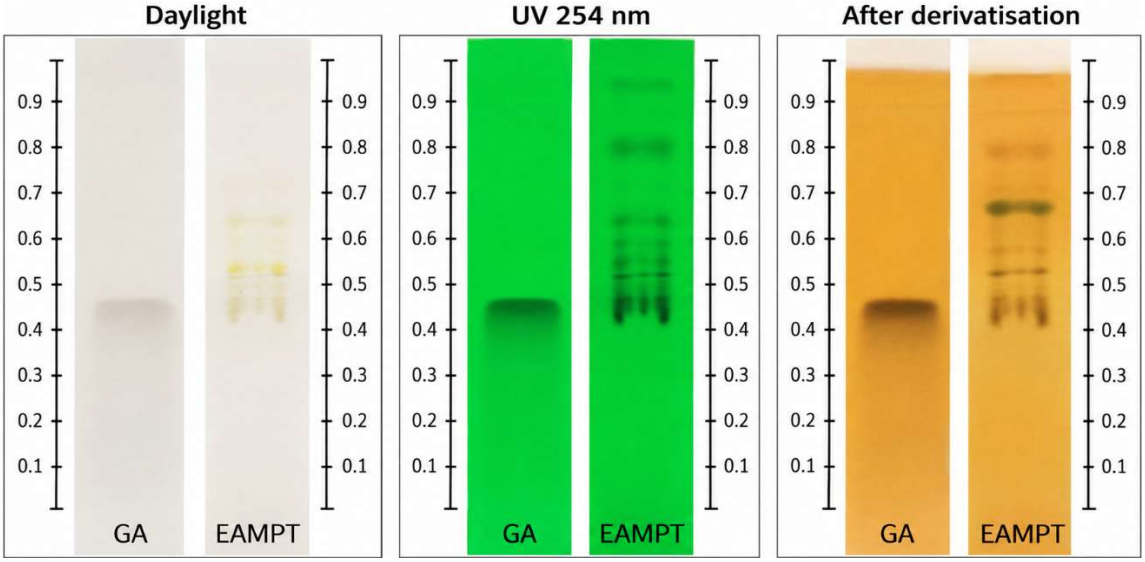


Figure 1. HPTLC chromatograms showing the bands of EAMPT and standard gallic acid (GA) photo documented before derivatisation under daylight, before derivatisation under UV 254 nm and after derivatisation with 2% ferric chloride.

Rosmarinic acid and procyanidin B1 were also isolated from the ethyl acetate fraction of *M. pendans* hydroethanolic extract at concentrations of 20.68 and 3.24 mg/g dry samples, respectively (Engida *et al.,* 2015). A phytochemical analysis of various solvent extracts of *M. rumphii* also indicated the presence of bioactive metabolites such as flavonoids and phenolics (Pasaribu *et al.,* 2018). Although there have been few studies on the phenolic profile of the genus Myrmecodia, the evidence presented here suggests that its phenolic content could affect the pharmacological applications of this plant, notably as an antioxidant and anti-inflammatory agent.

Plant phenolics are a diverse group of secondary metabolites with promising therapeutic applications in treating inflammation-related diseases. Phenolics are distinguished by the presence of one or more hydroxyl groups on aromatic rings, contributing to their antioxidant and anti-inflammatory properties. Phenolic compounds scavenge free oxygen radicals and activate the cellular antioxidant defence system, disrupting inflammatory signalling pathways, such as nuclear-factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPK). This inhibits the prolonged release of inflammatory mediators (Bucciantini *et al.,* 2021). Polyphenols have also been shown to modulate NO release and inhibit inflammatory enzymes involved in cellular metabolic pathways such as arachidonic acid and arginine metabolism (Hussain *et al.,* 2016).

Many polyphenols are known as effective antioxidants and anti-inflammatory agents, and curcumin is a classic example of a biologically active polyphenol. It is a beta-diketone molecule consisting of seven carbon chains and two modified aromatic rings, each with one hydroxy and methoxy group (Priyadarsini, 2014). Curcumin is often found in the plant rhizomes of the *Curcuma* genus (turmeric) and has many biological properties, including antimicrobial, anti-inflammatory, antioxidant, antirheumatic, cardioprotective, and neuroprotective activities (Sharifi-Rad *et al.,* 2020; Sun *et al.,* 2023). This serves as the basis for this study, in which medicinal plants rich in phenolic constituents have the potential to modulate cellular components, producing significant biological effects that could be leveraged as natural sources of novel pharmacological agents.

Cytotoxicity, antioxidant and anti-inflammatory effects of EAMPT in LPS-induced RAW 264.7 cells

Cell viability assay demonstrated a significant reduction in the viability of RAW 264.7 cells upon induction with 1 µg/mL LPS (p = 0.02) compared to the control group (Figure 2A).

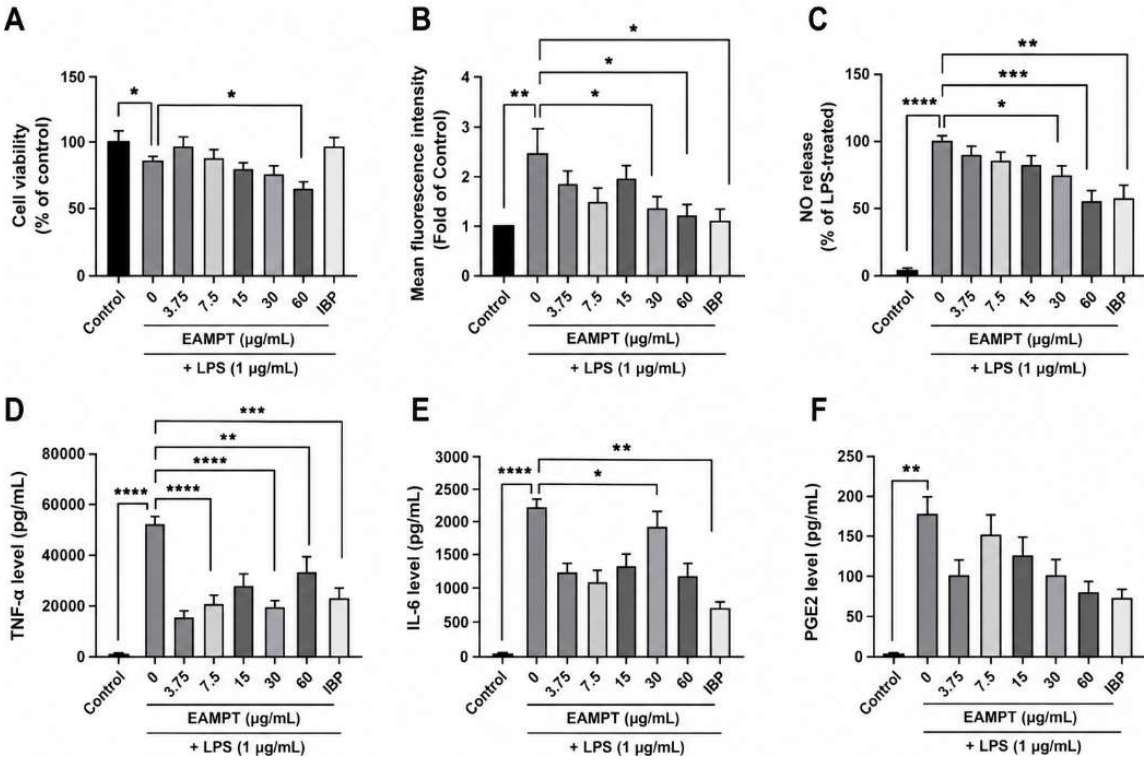


Figure 2. Effect of EAMPT on (A) cell viability and production of (B) ROS, (C) NO, (D) TNF-α, (E) IL-6 and (F) PGE2 in LPS-induced RAW 2647 cells. The RAW 264.7 cells were pre-treated with EAMPT (3.75, 7.5, 15, 30 or 60 μg/mL) or IBP (200 μM) for 1 h and stimulated with LPS for 24 h. Values are the mean ± SEM (n = 3), one-way ANOVA and Dunnett's post-hoc test: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (vs. LPS control group).

Treatment of LPS-induced RAW 264.7 cells with EAMPT of up to 30 μg/mL and IBP at 200 μM did not significantly affect cell viability. Meanwhile, at 60 μg/mL, EAMPT reduced the viability of LPS-induced RAW 264.7 cells by 17.8% (p = 0.0139). However, considering that 60 μg/mL is the IC_{50} value of EAMPT on normal RAW 264.7 cells, where the cells' growth was inhibited by 50% but showed increased cell viability to 65.1% upon induction with LPS, all concentrations of EAMPT up to 60 μg/mL were used in the subsequent experiments. LPS induction of RAW 264.7 cells resulted in a significant increase in intracellular ROS levels compared to the untreated group (2.37-fold, p < 0.01) (Figure 2B). Treatment of LPS-stimulated cells with EAMPT (30 and 60 μg/mL) and IBP was able to diminish the intracellular ROS levels by 1.15-fold, 1.25-fold, and 1.12-fold, respectively, compared to the LPS group (p < 0.05).

The anti-inflammatory effect of EAMPT was evaluated by measuring the LPS-induced NO release, and the maximum NO level was set to 100% to determine the percentage reduction of NO release by EAMPT (Figure 2C). Our results demonstrated a significantly lower NO level in the control group (2.48%), relative to the LPS-treated group (p < 0.0001). Pre-treatment of the LPS-induced cells with EAMPT at 30 and 60 μg/mL and IBP showed a significantly lower percentage of NO release at 71.04% (p = 0.013), 54.93% (p = 0.0003), and 59.8% (p = 0.0023), respectively, compared to the LPS-treated group. Moreover, LPS has significantly increased TNF-α levels (Figure 2D) compared to the control group (p < 0.0001). Pre-treatment of LPS-induced cells with EAMPT at all concentrations and IBP significantly inhibited TNF-α production compared to the LPS group. LPS induction strongly increased IL-6 levels (Figure 2E) compared to the control group (p < 0.0001). Pre-treatment with EAMPT at all concentrations did not significantly affect the IL-6 level, except at 30 μg/mL, where the IL-6 level was significantly

increased by 43.46% compared to the LPS-treated group ($p < 0.05$). Meanwhile, treatment with IBP has significantly diminished the IL-6 level by 50.63% compared to the LPS-treated group ($p = 0.009$). The production of PGE2 (Figure 2F) in the LPS-treated group was significantly increased compared to the control group ($p = 0.017$). However, there was no substantial change in PGE2 levels in LPS-induced cells treated with EAMPT or IBP.

The relationship between the effect of EAMPT on LPS-stimulated ROS generation and inflammatory markers in RAW 264.7 cells was evaluated to recognise a possible crosstalk effect between its antioxidant and immunomodulatory activities (Supplementary 2). A moderately positive correlation ($r = 0.5268$, $p = 0.0436$) was discovered between the effect of EAMPT on ROS and NO generation in LPS-induced RAW 264.7 cells. However, the ROS-inhibitory effects of EAMPT with TNF-α ($r = -0.05448$, $p = 0.8692$), IL-6 ($r = -008049$, $p = 0.7755$), and PGE2 ($r = -0.05448$, $p = .8471$) generation in LPS-induced RAW 264.7 cells were not statistically significant.

The present study employed LPS-induced RAW 264.7 cell culture as it is a well-established in vitro model to simulate the pathophysiology of inflammation-related disorders, which are characterised by elevated levels of oxidative stress and inflammatory markers (Facchin *et al.,* 2022). This corresponds with our results that the induction of murine macrophages with LPS at 1 µg/mL led to increased NO, TNF-α, IL-6, PGE2, and ROS relative to baseline values. Notably, pre-treatment of the LPS-induced RAW 264.7 cells with EAMPT decreased the inflammatory markers (NO, ROS, and TNF-α). The positive correlation between the ROS-inhibitory and NO-inhibitory effects of EAMPT suggests a synergistic and concurrent mechanism of *M. platytyrea* in simultaneously mitigating oxidative stress and inflammation. Several plants like white mulberry (*Morus alba* L.) and *Lasia spinosa* have demonstrated dual antioxidant and anti-inflammatory activities by reducing ROS, NO, and TNF-α production in the LPS-stimulated RAW 264.7 cell model (Nguyen *et al.,* 2020; Suriyaprom *et al.,* 2023).

EAMPT modulates gene and protein expression of cytokines and inducible enzymes in LPS-induced RAW 264.7 cells

Based on earlier results, the concentrations of EAMPT at 30 and 60 µg/mL were considered the effective concentrations producing the desired biological responses in this study. Hence, the effects of EAMPT at 30 and 60 µg/mL on LPS-induced gene expression of inflammatory cytokines and inducible enzymes in RAW 264.7 cells were examined using real-time qPCR (Figure 3). Activation of RAW 264.7 cells with LPS showed a significantly increased expression of the TNF-α gene compared to the control group ($p < 0.0001$). Treatment with EAMPT at 30 and 60 µg/mL and IBP strongly decreased the LPS-induced expression of the TNF-α gene by 28.25%, 38.76% and 21.17%, respectively ($p < 0.001$ and $p < 0.0001$). The IL-6 gene expression in LPS-induced RAW 264.7 cells was significantly increased compared to the control group ($p = 0.0171$). EAMPT at 30 µg/mL further increased the expression of the IL-6 gene in LPS-induced RAW 264.7 cells by 89.1% ($p = 0.0345$) compared to the LPS-treated group.

Regarding the inducible enzymes, LPS induction significantly increased the gene expression of iNOS compared to the control group ($p < 0.0001$). EAMPT at 30 and 60 µg/mL, as well as IBP, reduced iNOS gene expression significantly by 43.88% ($p = 0.0025$), 51.04% ($p = 0.0008$), and 57.48% ($p = 0.0003$), respectively, compared to the LPS-treated group. COX-2 gene expression was significantly increased in the LPS-treated group compared to the control group ($p = 0.0257$). EAMPT and IBP treatment did not affect COX-2 gene expression significantly, despite a modest upregulation. For the antioxidant enzyme HO-1, the LPS-treated group demonstrated a significantly higher HO-1 gene expression than the control group by 56.6% ($p = 0.0086$). Treatment with EAMPT at 60 µg/mL significantly increased HO-1 gene expression in LPS-induced RAW 264.7 cells by 57.2% compared to the LPS-treated group ($p = 0.0081$).

The protein expression of iNOS, COX-2 and HO-1 was analysed to understand the underlying mechanism of EAMPT in LPS-stimulated RAW 264.7 cells using Western blot. Our results (Figure 4) revealed significantly higher expression of iNOS (99%, $p < 0.0001$), COX-2 (99%, $p = 0.0002$) and HO-1 (72%, $p = 0.0042$) in the LPS control group compared to the untreated control group. Notably, EAMPT at both concentrations showed reduced expression of iNOS protein ($p < 0.0001$) compared to the LPS control group. Furthermore, LPS-stimulated cells treated with EAMPT at 60 µg/mL had increased HO-1 protein expression ($p < 0.0031$) compared to the LPS control group. However, COX-2 protein expression showed no substantial change.

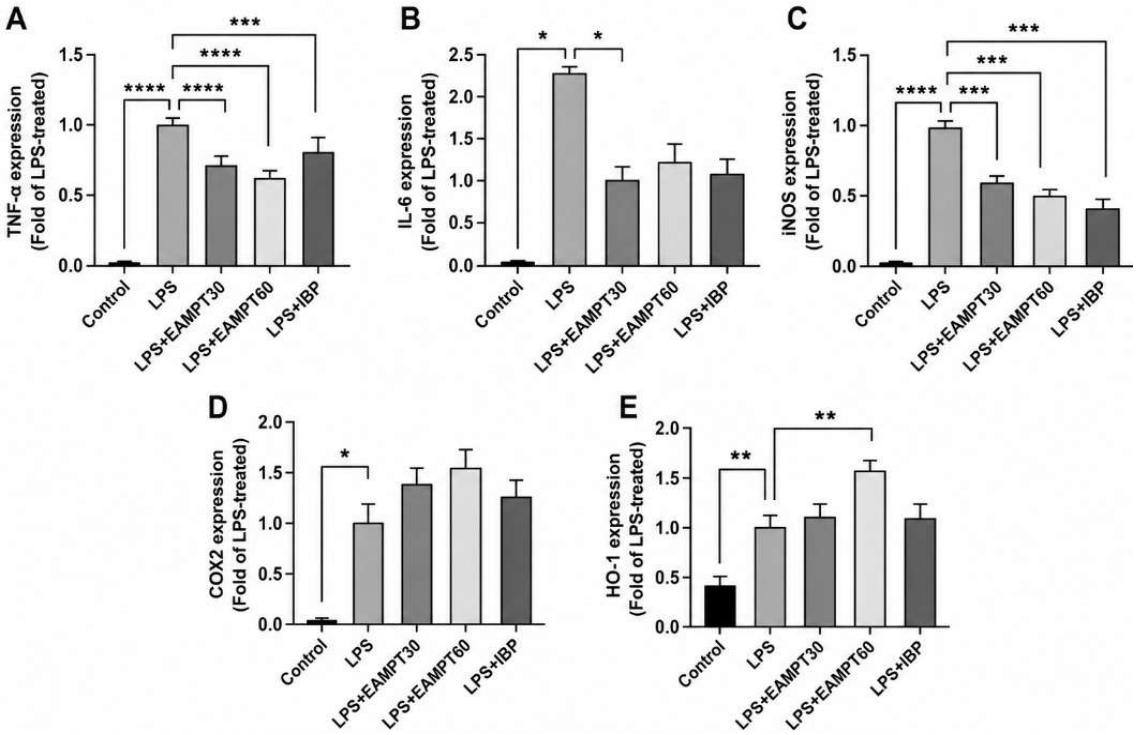


Figure 1. Effect of EAMPT on mRNA expression of inflammatory cytokines and inducible enzymes in LPS-induced RAW 264.7 cells. The RAW 264.7 cells were pre-treated with or without EAMPT or IBP for 1 h followed by exposure to LPS for another 4 h. Real-time qPCR was used to measure the mRNA expression of (A) TNF-α, (B) IL-6, (C) iNOS, (D) COX-2 and (E) HO-1. Results are presented as the mean fold expression of the LPS group (n = 3), one-way ANOVA and Dunnett's post-hoc test: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (vs. LPS).

Herein, EAMPT was shown to suppress the inflammatory mediators by inhibiting the iNOS gene and protein expression. This implies that EAMPT has therapeutic potential by mitigating iNOS/NO-mediated inflammation, an effective strategy for modulating the crosstalk with the antioxidant defence pathway. In addition, the antioxidant role of EAMPT was shown through its ability to upregulate the HO-1 gene and protein expression. HO-1 is a rate-limiting antioxidant enzyme that degrades heme into biliverdin, carbon monoxide, and free iron. It has an anti-inflammatory role that aids in tissue repair and the resolution of inflammation. Evidence has shown that induction of HO-1 during inflammation limits NO overproduction in macrophages through a negative feedback loop (Ashino *et al.,* 2008), which might explain the impact of *M. platytyrea* on the LPS-induced inflammation and oxidative stress in this study.

Interestingly, EAMPT-pre-treated LPS-induced RAW 264.7 cells expressed a greater IL-6 production, but no significant effect was observed on the PGE2 levels. This might be explained by multiple phytochemicals in the plant extract, each acting on a distinct mechanism. Studies have shown that the multi-constituent extract can modulate many cellular components crucial to the inflammatory response, whether at the protein or gene level (Merecz-Sadowska *et al.,* 2020). It could be that in this context, the various phytochemicals present in the plant extract may play different roles: as an immunostimulant by increasing IL-6, and as an immunosuppressant by diminishing TNF-α and NO, apart from its antioxidant effect that decreases ROS levels. It is worth noting that while IL-6 is regarded as a pro-inflammatory cytokine, it is a pleiotropic cytokine, playing a dual role against inflammation by stimulating or suppressing the cellular immune defence, depending on its environment.

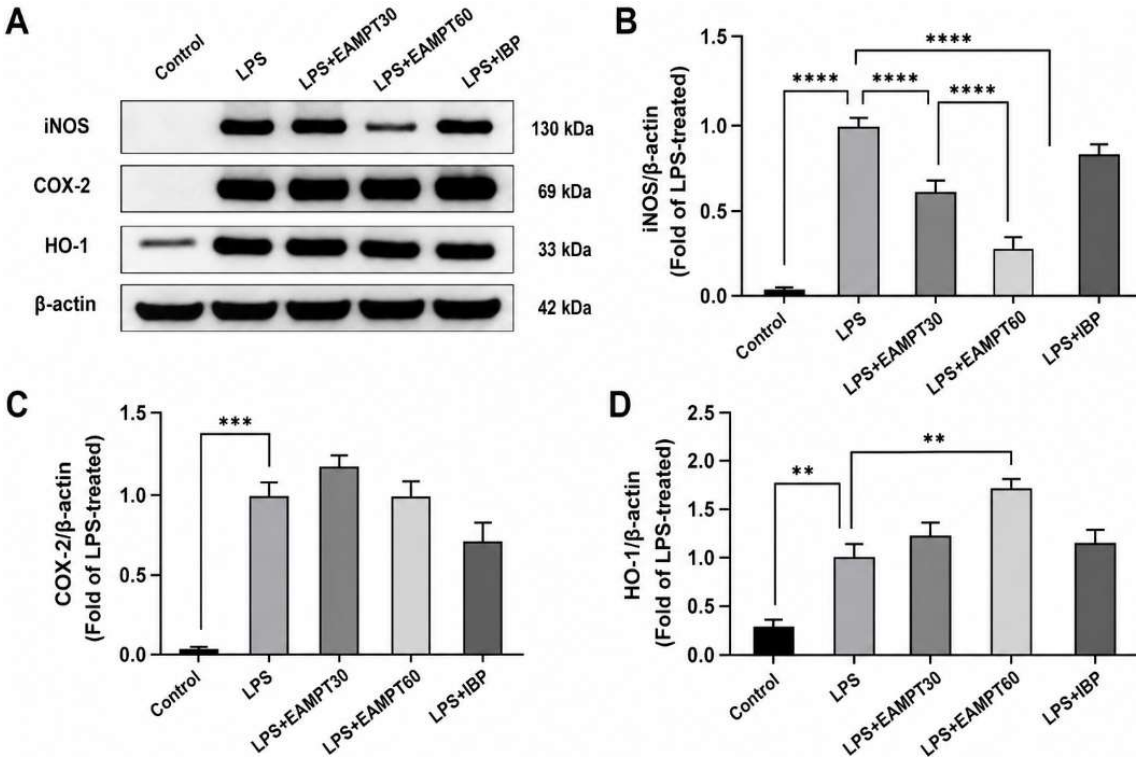


Figure 2. Effect of EAMPT on protein expression of inducible enzymes in LPS-induced RAW 264.7 cells. RAW 264.7 cells were pretreated with or without EAMPT (30 or 60 μg/mL) or IBP for 1 h, followed by exposure to LPS for an additional 24 h. (A) Representative blot image showing the bands of iNOS, COX-2, HO-1 and β-actin with their respective molecular weight. Graphs showing the relative protein expression of (B) iNOS, (C) COX-2 and (D) HO-1. Values are the mean fold expression of the LPS group (n = 3), one-way ANOVA and Dunnett's post-hoc test: **p < 0.01, ***p < 0.001, ****p < 0.0001 (vs. LPS).

For instance, in Coronary Virus Disease (COVID)-19 infection, IL-6 exhibited a protective role as an antimicrobial and an anti-inflammatory agent, resulting in increased macrophage survival and improved host immune response. Studies have reported that IL-6 inhibition by tocilizumab, an IL-6 receptor antagonist, among COVID-19 patients has caused higher risks of opportunistic infections, including pneumonia, which might complicate the disease further (Scherger *et al.,* 2020). This suggests that IL-6 does play a substantial adaptive immunological role in enhancing host immune response against infection and inflammation. Hence, EAMPT's effect on IL-6 may not be purely anti-inflammatory, but as an immunostimulatory agent that facilitates macrophage survival against acute inflammatory responses.

Macrophage polarisation also plays a vital role as LPS-activated macrophages are polarised to the M1 type, which is considered pro-inflammatory, whereas M2 macrophages are anti-inflammatory and are abundant in the tumour microenvironment, producing copious amounts of IL-6 to help balance the inflammatory response (Hirano, 2021). In an in vitro experiment, Aro et al. (2019) reported that *Cremaspora triflora* and *Psychotria zombamontana* (Rubiaceae) extracts significantly enhanced TNF-α and interferon-gamma (IFN-γ) production, respectively, in an LPS-induced human monocytic (U937) cell line compared to the untreated LPS-induced cells, signifying their capacity to stimulate the immune system (Aro *et al.,* 2019). Furthermore, research on the immunomodulatory effects of selected South African medicinal plants revealed that aqueous extracts of *Plumbago auriculata* (Plumbaginaceae), *Warburgia salutaris* (Canellaceae) and *Zantedeschia albomaculata* (Araceae) promote IL-6

release in LPS-induced RAW 264.7 cells (Khumalo *et al.,* 2024). These findings support our discoveries that plant extracts, particularly those from the Rubiaceae family, can boost cytokine release under stress conditions, indicating their potential effect as an immunostimulant, besides their antioxidant and anti-inflammatory properties. Several other Rubiaceae plants, namely *Uncaria*, *Morinda*, and *Coffea* genus, have been shown to regulate ROS and inflammatory markers, resulting in anti-inflammatory, antihypertensive, antihyperglycemic, and lipid-lowering effects for treating metabolic syndrome and cardiovascular diseases (González-Castelazo *et al.,* 2023).

Nevertheless, the absence of a substantial effect on the COX-2/PGE2 pathway, a component of arachidonic acid metabolism (McEvoy & Pirmohamed, 2021), suggests that *M. platytyrea* might exert a selective action on the iNOS/NO pathway. Hence, the results obtained from this study further strengthen our understanding of the mechanisms of the multi-constituent plant extract that acts on both protein and gene levels with selective targets of multiple signalling pathways. Compared to single-lead compounds that target only one component of a single disease, it could be that mixtures of bioactive compounds act synergistically towards regulating the cellular immune response against triggering pathogens or injurious agents during an acute inflammatory state. The growing interest in traditional medicine is most likely due to the unique ability of these natural bioactive compounds to modulate the immune system, either through additive effect (where the combined action of two chemicals results in an equal sum of the chemicals acting alone) or synergistic effect (where the resulting action of two chemicals is more significant than their independent effect).

With an increasing research interest in phytomedicine, many preclinical and clinical studies are being done to explore the pharmacological potential of medicinal plants as natural immunomodulators, whether in their extract forms or as isolated compounds (Alhazmi *et al.,* 2021; Memarzia *et al.,* 2021; Tharakan *et al.,* 2021). Some of the commonly used natural active ingredients in traditional medicine for their immunomodulatory activities include quercetin, resveratrol, andrographolide, curcumin, capsaicin, galangin, aloe-emodin, asiatic acid, glabridin, celastrol, and icariin (Jantan *et al.,* 2015; Sai Priya *et al.,* 2024).

In line with our study, epigallocatechin gallate (EGCG), a green tea polyphenol, was shown to inhibit TNF-α and iNOS/NO expression against LPS-induced inflammation in macrophages and neutrophils. Additionally, EGCG inhibited the inhibitor kappa-B alpha (IκB-α) phosphorylation, thus preventing nuclear translocation of the active NF-κB, abrogating the inflammatory response (Wang *et al.,* 2021). Quercetin is also a widely studied polyphenol with an immunomodulatory role. Studies have demonstrated that quercetin diminished pro-inflammatory cytokines such as TNF-α, IL-1β and IL-8 in various cellular models of lung inflammation, including cigarette smoke-induced A549 alveolar epithelial cells and LPS-induced mouse alveolar macrophages. Quercetin was found to act on different inflammatory signalling pathways, including the NF-κB and MAPK pathways (Bernini & Velotti, 2021). These findings could serve as the theoretical basis for deciphering the immunomodulatory role of *M. platytyrea*, highlighting its potential therapeutic benefits correlating to its substantial phenolic content. Nevertheless, the effect of *M. platytyrea* on upstream inflammatory signalling pathways such as the NF-κB and MAPK remains to be explored in the future. According to the literature, other *Myrmecodia* sp., such as *M. pendans* and *M. tuberosa* flavonoid-rich extracts, had an inhibitory effect on the NF-κB signalling pathway in oral cancer cells, resulting in increased apoptosis (Achmad *et al.,* 2016; Yuletnawati *et al.,* 2016).

Therefore, considering the results of this study, we established that EAMPT has an immunomodulatory impact through complex mechanisms, regulating both the inflammatory and antioxidant pathways in an LPS-induced RAW 264.7 cell line inflammation model. EAMPT simultaneously reduces pro-inflammatory markers like iNOS and TNF-α while upregulating HO-1, the antioxidant enzyme. As such, EAMPT, comprising mixtures of bioactive compounds including phenolics, could offer a holistic approach to preventing inflammatory and oxidative stress responses from escalating to pathological states. Moreover, there was no inhibition of the arachidonic acid pathway via COX, which might have resulted in serious complications such as gastrointestinal bleeding due to suppressed prostacyclin synthesis, as commonly seen with ibuprofen and other COX inhibitors (McEvoy & Pirmohamed, 2021). Collectively, the diverse phytochemicals present in *M. platytyrea* are

postulated to exert synergistic effects that surpass those of single-compound interventions, particularly in modulating intricate cellular signalling networks. This multifaceted pharmacological activity is pivotal for restoring and maintaining homeostatic cellular physiology under pathophysiological conditions.

CONCLUSION

In conclusion, our study has demonstrated the immunomodulatory role of *M. platytyrea* by regulating LPS-induced inflammation and oxidative stress markers at both the gene and protein levels. The extract diminished iNOS/NO, TNF-α, and ROS levels while enhancing IL-6 and HO-1 expression. This promising therapeutic effect could be attributed to the significant total phenolic content of the ethyl acetate tuber extract, which could impact the key inflammatory and antioxidant signalling pathways. Therefore, more research is needed to characterise the phytochemical profiles of *M. platytyrea* and decipher its complex mechanism of action, towards developing it as an immunomodulatory agent for treating inflammatory diseases.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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